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Na^+,K^+-ATPase activity is selectively increased in thalamus in thiamine deficiency prior to the appearance of neurological symptoms

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Abstract

The relationship between progression of neurological status and the activities of both Na^+,K^+- and Mg^{2+}-dependent-ATPase (adenosine 5'-triphosphate phosphohydrolase) was investigated in brain regions of pyrithiamine-induced thiamine deficient rats. Thalamic Na^+,K^+-ATPase activity was selectively increased by 200% ($P < 0.01$) prior to the appearance of symptoms of thiamine deficiency and normalized in symptomatic rats. This selective transitory activation precludes a mediation by brain soluble fraction Na^+,K^+-ATPase modifiers as does the unaltered distribution in regional high-affinity [^{3}H]ouabain binding densities observed throughout the time-course used in these experiments. Na^+,K^+-ATPase maintains cellular ionic gradients and has been implicated in neurotransmitter uptake and release mechanisms. The fact that the increased thalamic Na^+,K^+-ATPase activity coincides with the early alterations in serotonin metabolism observed in similarly treated animals and the concomitantly early increase in glucose utilization previously observed in the thalamus of thiamine-deficient rats is discussed.

Keywords: Thiamine deficiency; ATPase; Thalamus; (Rat); Pyrithiamine; [^{3}H]Ouabain

1. Introduction

Thiamine (vitamin B_1) deficiency, as with most metabolic and toxic insults to the central nervous system, results in selective vulnerability of brain structures with sparing of neighbouring ones. The basis of this selectivity in the case of thiamine deficiency is unknown. Previous studies, using the central thiamine antagonist pyrithiamine (1-[(4-amino-2-methyl)-5-pyrimidylmethyl]-2-methyl-3-[β-hydroxyethyl]pyridinium bromide) to induce thiamine deficiency in the rat, have demonstrated, among other events, neurological damage to midline thalamic structures (see Butterworth, 1986) and altered biogenic amine uptake/release (Plaitakis et al., 1982). Recent autoradiographic evidence of region selective increases in postsynaptic 5-HT_{2A} receptor densities coincides with decreases in levels of the biogenic amine neurotransmitter serotonin (5-hydroxytryptamine; 5-HT) in experimental thiamine deficiency prior to the appearance of neurological symptoms (Mousseau, Rao and Butterworth, submitted). Furthermore, denervation with the indoleamine neurotoxin 5,6-dihydroxytryptamine has been shown to result in a hypersensitivity-like response of Na^+,K^+-ATPase activity to the 5-HT receptor agonist quipazine, which can be prevented by the 5-HT receptor antagonist methysergide, suggesting a postsynaptic 5-HT receptor-mediated response (Hernández, 1982).

Na^+,K^+-ATPase (adenosine 5'-triphosphate phosphohydrolase, EC 3.6.1.3), an integral membrane protein responsible for the active transport of Na^+ and K^+ ions, controls the delicate chemical gradient vital for optimal function of most mammalian cells including those of the central nervous system. In the central nervous system, Na^+,K^+-ATPase has been implicated in the generation of electrical potentials (Bonting et al., 1961; Fahn and Cote, 1968) and in neurotransmitter uptake and release mechanisms (Meyer and Cooper, 1984). In view of our recent findings of decreased levels of 5-HT in experimental thiamine deficiency and the suggested role of Na^+,K^+-ATPase in the neurotransmitter release process, we undertook to assess the effects of pyrithiamine-induced thiamine deficiency on ATPase activity in various brain structures, including tha-

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lamus, of the rat and its relation to the progression of neurological symptoms. Furthermore, the distribution of the functional enzyme was obtained by visualizing binding sites using in vitro autoradiography of [^{3}H]ouabain which labels the functional phosphorylated high-affinity α_2 and α_3 isoforms but not the low-affinity α_1 isoform in the rat (Sweadner, 1989).

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (~ 225 g) were housed individually under constant temperature (21–22°C) and humidity, and on a 12 h : 12 h light/dark cycle (lights on at 6:00 a.m.). Animals were used strictly in accordance with the guidelines of the University of Montreal Animal Care Committee and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHEW publication (N.I.H.) 78–23, revised 1978].

2.2. Treatment groups

Rats were made thiamine deficient as described elsewhere (Rao et al., 1995). Briefly, rats were randomly assigned to treatment groups: Group 1 (*presymptomatic*) rats received a thiamine-deficient diet (vitamin-free casein supplemented with all vitamins except thiamine) in addition to pyrithiamine (500 μg/kg/day) for 7 days. No evidence of neurologic abnormalities (e.g. abnormal gait and righting reflexes) was present in any of the rats at this time; group 2 (*symptomatic*) rats also received a thiamine-deficient diet and pyrithiamine. However, treatment was continued until loss of righting reflex was evident, generally between days 12 and 14; group 3 (*pair-fed control*) rats received the thiamine-deficient diet paired to equal the food consumption of rats in groups 1 and 2, above. In addition, these rats received daily injections of thiamine (100 μg/kg). Half of the control rats were killed on day 7 along with the corresponding pair-fed presymptomatic rat. The remainder of the control rats were killed on the same day as the corresponding pair-fed symptomatic rat.

2.3. Tissue collection

Rats were decapitated and the brain (meninges and pineal gland removed) were quickly removed (maximum 15 s postmortem) and separated mid-sagittally. One half was immersed in isopentane cooled over dry ice and then stored at −80°C until needed. A series of 20 μm sections was obtained from each brain using a cryomicrotome (−20°C). These were thaw-mounted onto gelatin-coated glass slides and were stored at −20°C until the autoradiographic study (4–6 weeks). The other half of the brain was immersed in ice-cold saline and then dissected on ice. The frontal cortex (a 1 mm deep coronal section at the rostral portion of the cortex) and the cerebellum were easily separated from the rest of the brain. The striatum consisted mainly of the head of the caudate nucleus. The thalamus was found between the anterior and posterior commissure, just below the hippocampus/dentate gyrus. All tissue was frozen at −80°C until time of assay (within 4 weeks).

2.4. Assay for Na^+,K^+-ATPase activity

The activity of Na^+,K^+-ATPase was assayed as ouabain-sensitive ATP hydrolysis (Bonting et al., 1961). Brain tissues were homogenized in 20–40 vols. of deionized water and frozen for at least 24 h. Total ATPase activity was determined in 30 mM Tris buffer (pH 7.4 containing 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$ and 3 mM ATP as substrate), whereas Mg^{2+}-dependent ATPase activity was determined in the absence of NaCl and KCl, and in the presence of 1 mM ouabain. Blanks were run in the absence of salts and ATP. The reaction, carried out at 37°C, consisted of a 10 min preincubation and, following addition of ATP to the appropriate tubes, a 15 min incubation period. The reaction was terminated by addition of trichloroacetic acid (10% w/v). Following centrifugation, equivolumes of the supernatant and colouring reagent (400 mg ferrous sulphate per 10 ml of 1% ammonium molybdate in 1.15 N H_2SO_4) were mixed and left to stand at room temperature for 15 min at which time the liberated inorganic phosphate was estimated by spectophotometry (λ = 700 nm). Na^+,K^+-ATPase activity was defined as the difference between total ATPase and Mg^{2+}-dependent-ATPase activities. Protein determinations were carried out using the Folin phenol reagent method (Lowry et al., 1951).

2.5. [^{3}H]Ouabain quantitative autoradiography

The binding protocol of Brines et al. (1991) was used to determine high-affinity [^{3}H]ouabain binding density. Briefly, tissue sections were washed in Tris buffer (50 mM, 10 mM $MgCl_2$, pH 7.4) at 27°C for 15 min to dissociate any bound endogenous substances. The sections were then incubated at room temperature for 45 min in 50 mM Tris buffer containing 10 mM NaCl, 5 mM ATP and 10 nM [^{3}H]ouabain. Non-specific binding was determined in the presence of 1 mM unlabeled ouabain. Following incubation, the slides were washed twice in buffer for 5 min at 4°C, dipped in deionized water to remove buffer salts, and then rapidly air-dried. Autoradiograms were prepared by apposing labeled sections together with a tritium standard to Hyperfilm-^{3}H for 5 days. Tissue concentrations of [^{3}H]ouabain were measured by quantitative densitometry analysis using a MCID computerized image-analysis system (Imaging Research, St. Catharine's, ONT,

Canada) which provides relative density values in nCi/mg tissue. These were converted to values in pmol/mg tissue by factoring in the specific activity of the radioligand. Specific binding was calculated by subtracting the non-specific binding and background film density from the total binding. Brain nuclei were identified from plates 45 to 47 of the rat brain atlas of Paxinos and Watson (1982). To differentiate brain sections located at 0.4 mm lateral from 0.9 mm lateral, the structural morphology of the hippocampal CA1 region was employed (e.g. at 0.4 mm lateral the CA1 region is caudal to the CA3 region and the CA2 region is absent; at 0.9 mm lateral the CA1 region is superior to the CA2 region). In addition, the fasciculus retroflexus from the lateral habenular nucleus is only visualized at 0.9 mm lateral. At 1.4 mm lateral neither the habenula nor the fasciculus retroflexus remain visible whereas the appearance of the [striated] caudate nucleus is striking. Further structural confirmation was found with the pyramidal tracts which, being located medially, are absent by 1.4 mm lateral. Intra- and interjudge reliability of image analysis and neuroanatomical structure verification was assessed on randomly selected autoradiograms. Correlation (Pearson's) coefficients for intra- and interjudge analyses were $r = 0.94$ and $r = 0.92$, respectively.

2.6. Materials

The thiamine-deficient diet was purchased from ICN, Nutritional Biochemicals (Cleveland, OH, USA). Pyrithiamine hydrobromide, thiamine hydrochloride, adenosine 5'-triphosphate (ATP), Tris-(hydroxymethyl)aminomethane (Tris), ammonium molybdate, ferrous sulfate and the remainder of the salts were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ouabain (G-strophantidin) was purchased from Boehringer Mannhein, Mannheim, Germany. Ouabain-[^{3}H(G)] (spec. act.: 20.5 Ci/mmol) was purchased from New England Nuclear Research Products (Mississauga, ONT, Canada).

2.7. Statistics

Effect of treatment was determined by analysis of variance followed by multiple comparisons of the means using the post-hoc Tukey test. $P < 0.05$ was defined as the criterion for statistical significance.

3. Results

The effect of pyrithiamine-induced thiamine deficiency on frontocortical, thalamic, striatal, and cerebellar ATPase activities is depicted in Fig. 1. Of the regions examined, only the thalamus presented a significant increase ($\sim +200\%$) in the activity of Na^+,K^+-ATPase, and this only in the presymptomatic group. There was no difference in Mg^{2+}-dependent-ATPase activity in either presymptomatic or symptomatic thiamine-deficient rat brain regions. An extensive quantitative autoradiographic study of the regional distribution of [^{3}H]ouabain binding was undertaken, thus allowing for comparison with published results and confirmation of binding specificity (a representative sagittal section is presented in Fig. 2); however, for ease of interpretation and data presentation only a selected list of brain nuclei known or thought to be involved in thiamine deficiency is given. This study did not reveal any changes in the binding density of [^{3}H]ouabain in any of the regions of thiamine-deficient rat brains (Table 1).

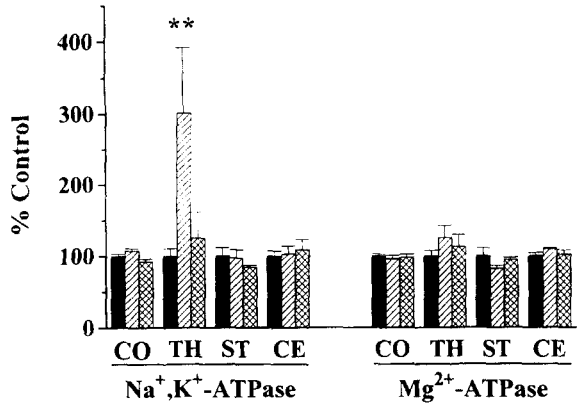

Fig. 1. Regional activities (% control ± S.E.M.) of Na^+,K^+-ATPase and Mg^{2+}-dependent ATPase in brain of pyrithiamine-treated rats. Control values (solid bars) are based on $n = 10$. Thiamine-deficient animals received a thiamine-deficient diet and daily injections of pyrithiamine (500 μg/kg). Presymptomatic rats (hatched bars, $n = 6$) were killed prior to the appearance of neurological symptoms. Symptomatic rats (cross-hatched bars, $n = 6$) were killed immediately upon appearance of neurological symptoms such as loss of righting reflex. CO: frontal cortex; TH: thalamus; ST: striatum; CE: cerebellum. Control values for Na^+,K^+-ATPase (μmol/h/mg protein) in CO: 9.2 ± 0.3; TH: 4.8 ± 0.5; ST: 5.5 ± 0.6; CE: 11.4 ± 0.8. Control values for Mg^{2+}-ATPase (μmol/h/mg protein) in CO: 9.0 ± 0.2; TH: 12.7 ± 1.0; ST: 19.0 ± 2.0; CE: 15.0 ± 0.6. ** Significantly different ($P < 0.01$) from control values.

4. Discussion

Our choice of pyrithiamine as a means of inducing thiamine deficiency in experimental animals was based on the similarity in changes and/or distribution in neurological symptoms and of neuropathological lesions (e.g. in thalamic nuclei and mammillary bodies) in this model and the clinical syndrome in humans most often associated with chronic thiamine deficiency, i.e. the Wernicke-Korsakoff syndrome (see Butterworth, 1986). The control ATPase activity values of the present study are in good agreement with literature values (Matsuda and Iwata, 1987). Thiamine deficiency induced a selective 3-fold increase in Na^+,K^+-ATPase activity in the thalamus (Fig. 1). Furthermore, the increase was apparent prior to the appearance of neurological symptoms of thiamine deficiency. Activity of Mg^{2+}-dependent ATPase was un-

changed in all brain regions of pyrithiamine-treated rats. An earlier study using dietary thiamine deprivation alone to produce thiamine deficiency did not reveal any significant changes in Na^+,K^+-ATPase activity (Matsuda and Iwata, 1987); however, enzyme activity in the thalamus, a structure which appears not to be vulnerable in this dietary model of thiamine deficiency, was not measured. Other studies have demonstrated an inhibitory effect of pyrithiamine in vitro on Na^+,K^+-ATPase activity in brain preparations (Matsuda et al., 1984). This in vitro inhibitory effect is in contrast to the results of the present ex vivo study. The existence of a soluble brain fraction able to affect ATPase activity has also been demonstrated (Rodríguez de Lores Arnaiz, 1993 and references therein). This fraction can be separated by gel filtration into two subfractions; peak I stimulates both Na^+,K^+- and Mg^{2+}-dependent ATPases, and peak II inhibits Na^+,K^+-ATPase alone. As we observed an increase in Na^+,K^+-ATPase alone, we can assume that neither peak I nor peak II modulated the present transitory effect on Na^+,K^+-ATPase activity. This is corroborated by the present findings of unaltered high-affinity [^{3}H]ouabain binding which is also sensitive to these same Na^+,K^+-ATPase modifiers (Antonelli et al., 1991). Interestingly, the dietary thiamine deprivation model used by Matsuda and Iwata (1987) did result in slight, yet significant, regionally selective decreases in high-affinity ouabain binding even though Na^+,K^+-ATPase activity in these same regions remained unchanged. The protocol used for visualization of [^{3}H]ouabain binding provides information on the high-affinity α_2 and α_3 isoforms but not on the low-affinity α_1 isoform (Sweadner, 1989; Brines et al., 1991). Two possible explanations, based on the present series of experiments, may account for the observed increase in thalamic Na^+,K^+-ATPase activity. (i) Activation of the enzyme, independently of an α_2 or α_3 isoform-mediated event, may explain this phenomenon or, alternatively, (ii) the increase in activity may result from activation of the α_1 isoform, thereby implicating glial Na^+,K^+-ATPase in this effect.

Consistent with the findings of the present study, cerebral glucose utilization, assessed using the 2-deoxyglucose autoradiographic method is paradoxically increased at presymptomatic stages of thiamine deficiency in several brain regions including thalamus (Hakim and Pappius, 1983). The reason for this early increased brain glucose

Table 1
Distribution of high-affinity [^{3}H]ouabain binding in thiamine-deficient rat brain as determined by quantitative autoradiography

Structure	Control	Presymptomatic	Symptomatic
Cerebral cortex			
Frontal (area 10)	1.59 ± 0.22	1.68 ± 0.25	1.63 ± 0.27
Frontoparietal motor (area 2)	1.74 ± 0.17	1.74 ± 0.27	1.68 ± 0.12
Anterior cingulate (area 24)	1.66 ± 0.16	2.09 ± 0.24	1.65 ± 0.05
Anterior olfactory nucleus	1.71 ± 0.13	1.72 ± 0.24	1.69 ± 0.07
Caudate putamen	1.51 ± 0.18	1.53 ± 0.14	1.37 ± 0.19
Nucleus accumbens	1.49 ± 0.20	1.47 ± 0.11	1.29 ± 0.13
Hippocampus			
CA1 field	1.91 ± 0.11	1.99 ± 0.19	1.97 ± 0.20
CA2 field	2.00 ± 0.13	1.99 ± 0.23	2.02 ± 0.16
CA3 field	1.59 ± 0.16	1.64 ± 0.14	1.57 ± 0.04
Thalamus			
Anteromedial nucleus	1.54 ± 0.32	1.74 ± 0.37	1.76 ± 0.15
Mediodorsal nucleus	1.70 ± 0.27	1.95 ± 0.31	1.93 ± 0.13
Centromedial nucleus	1.61 ± 0.34	1.86 ± 0.23	1.96 ± 0.15
Gelatinosum	1.55 ± 0.32	1.89 ± 0.24	1.81 ± 0.17
Centrolateral nucleus	1.36 ± 0.13	1.57 ± 0.32	1.78 ± 0.17
Ventrolateral nucleus	1.30 ± 0.14	1.43 ± 0.26	1.45 ± 0.23
Anteroventral nucleus	1.32 ± 0.12	1.61 ± 0.24	1.73 ± 0.13
Hypothalamus			
Ventromedial nucleus	1.80 ± 0.32	1.86 ± 0.21	1.86 ± 0.13
Dorsomedial nucleus	1.61 ± 0.26	1.90 ± 0.26	1.79 ± 0.24
Inferior colliculus	2.06 ± 0.21	2.14 ± 0.16	2.31 ± 0.14
Dorsal raphe nucleus	2.27 ± 0.37	2.28 ± 0.05	2.55 ± 0.24
Locus coeruleus	2.09 ± 0.40	2.16 ± 0.46	2.44 ± 0.09
Inferior olivary nucleus	2.03 ± 0.30	1.96 ± 0.11	2.04 ± 0.29
Cerebellum			
Molecular layer	1.46 ± 0.29	1.54 ± 0.06	1.49 ± 0.10
Granular layer	2.04 ± 0.30	1.92 ± 0.20	2.13 ± 0.11

Rats received a thiamine-deficient diet and thiamine (100 μg/kg: control group, $n = 9$) or pyrithiamine (500 μg/kg: presymptomatic ($n = 4$) and symptomatic ($n = 5$) thiamine-deficient groups). Each value (pmol/mg tissue) represents the mean ± S.E.M.

Fig. 2. A representative autoradiogram of the distribution of specific high-affinity [^{3}H]ouabain binding in control rat brain (sagittal aspect). The binding distribution of these sites was unaltered in thiamine-deficient animals (i.e. following 7 or 14 days treatment with a thiamine-deficient diet and daily injections of pyrithiamine, 500 μg/kg; see Table 1).

utilization is unknown but could relate to the early decreases in activity of α-ketoglutarate dehydrogenase in thalamus at presymptomatic stages of thiamine deficiency (Butterworth and Héroux, 1989). Since α-ketoglutarate dehydrogenase is rate-limiting for cerebral glucose utilization, ATP levels could be maintained transiently by increased glycolysis (the 'Crabtree Effect'), accompanied by transiently increased brain glucose utilization. Na^+,K^+-ATPase, via its role in the maintenance of membrane potentials, accounts for as much as 25% of cerebral oxidative metabolism (Keesey and Wallgren, 1965). Na^+,K^+-ATPase may be also involved in neurotransmitter release and deactivation (Meyer and Cooper, 1984): inhibition of Na^+,K^+-ATPase activity is thought to result in neurotransmitter release. The present finding of increased Na^+,K^+-ATPase activity could explain the altered 5-HT metabolism in the thalamus of pyrithiamine-induced thiamine deficient rats (Mousseau, Rao and Butterworth, submitted).

The findings in the present study are consistent with previous reports of increased thalamic glucose utilization, altered neurotransmitter availability and the observed selective thalamic neuronal cell loss in pyrithiamine-induced thiamine deficiency. The exact sequence of events (and the direction of causality) in this relationship, as well as the significance of each, remain to be determined. That these events involve the thalamus in one aspect or another infers a role for each event in the pathogenesis of thalamic neuronal loss in thiamine deficiency.

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